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# CERKL interacts with mitochondrial TRX2 and protects retinal cells from oxidative stress-induced apoptosis



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## ABSTRACT

Mutations in the *ceramide kinase-like* gene (*CERKL*) are associated with severe retinal degeneration. However, the exact function of the encoded protein (*CERKL*) remains unknown. Here we show that *CERKL* interacts with mitochondrial thioredoxin 2 (*TRX2*) and maintains *TRX2* in the reduced redox state. Overexpression of *CERKL* protects cells from apoptosis under oxidative stress, whereas suppressing *CERKL* renders cells more sensitive to oxidative stress. In zebrafish, *CERKL* protein prominently locates in the outer segment and inner segment of the photoreceptor of the retina. Knockdown of *CERKL* in the zebrafish leads to an increase of retinal cell death, including cone and rod photoreceptor degeneration. Signs of oxidative damage to macromolecules were also detected in *CERKL* deficient zebrafish retina. Our results show that *CERKL* interacts with *TRX2* and plays a novel key role in the regulation of the *TRX2* antioxidant pathway and, for the first time, provides an explanation of how mutations in *CERKL* may lead to retinal cell death.

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## 1. Introduction

Vertebrate vision is mediated by two classes of photoreceptors, rods and cones. Rods are sensors of dim light and cones of color and high acuity vision. Retinal degenerations that involve photoreceptor loss, including cone-rod dystrophy (CRD), retinitis pigmentosa (RP), and

age-related macular degeneration (AMD), are major causes of blindness worldwide. In these disorders, protracted cone death is often the last stage in the disease course [1–3]. Accordingly, cone survival is a major target of therapeutic intervention. Several animal models of retina degeneration show extensive signs of oxidative damage in the retina [4–6]. However, the role of oxidative stress and its causal link to a specific genetic defect have not been demonstrated previously.

Identified and cloned in 2004, *CERKL* was reported by several groups as one of the causative genes associated with autosomal recessive RP [7], including our study of a nonconsanguineous Chinese family [8]. In 2009, Tomas et al. observed that *CERKL* mutations were associated with widespread retinal degeneration with prominent early maculopathy; hence, the clinical presentation was more akin to that of autosomal recessive CRD [9]. In patients, they cause widespread retinal degeneration with early and prominent loss of macular cone and rod photoreceptors [7,9,10].

To date, eight disease-causing mutations in *CERKL* have been identified; there are two missense mutations (R106S and C125W) and the others are null or splice-site mutations [7–12]. The functional and anatomical defects resulting from mutations in *CERKL* are consistent

**Abbreviations:** CoCl<sub>2</sub>, cobalt(II) chloride; DAPI, 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride; dpf, days post fertilization; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GST, glutathione S-transferase; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; IgG, immunoglobulin G; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; siRNA, small interfering RNA; TUNEL, terminal dUTP nick end-labeling

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with the expression pattern of CERKL in the mouse retina; in the outer retina, it is mainly expressed in cone photoreceptors and in the inner retina, weakly in amacrine cells and ganglion cells [13,14].

Previous studies of the function and disease mechanism of CERKL have focused on the metabolism of sphingolipid and ceramide, both in cell culture and in mice [15–17]. However, these properties of CERKL remain obscure. Recently, it was demonstrated that CERKL interacts with several neuronal calcium sensor proteins in the retina [18], however, the functional significance and consequences of these interactions are not clear.

The thioredoxin system is composed of thioredoxin (TRX), thioredoxin reductase (TrxR) and NADPH. Of the two major members in the mammalian thioredoxin superfamily, TRX1 and TRX2, TRX2 is mitochondria-specific and a critical regulator of redox balance that is required for cell viability [19–22]. Hypoxia-induced apoptosis can be ameliorated by modulating TRX2 [23].

To investigate the physiologic role that CERKL plays in the retina and its link to photoreceptor apoptosis, we screened for CERKL-interacting proteins. Here we show that CERKL can localize to mitochondria, where it interacts with TRX2 and acts as a novel player in the regulation of the mitochondrial peroxiredoxin-mediated antioxidant pathway. In NIH3T3 cells and zebrafish, we found that CERKL protects cells from apoptosis by maintaining the reduced state of mitochondrial TRX2. These data illuminate a novel mechanism for CERKL mutations that causes redox system dysfunction and retinal cell death.

## 2. Materials and methods

### 2.1. Reagents and antibodies

CoCl<sub>2</sub> was purchased from Sigma. Antibodies used in this study include: CERKL, tubulin and GAPDH (Abcam); Myc (Sigma); GFP, flag, and GST (Proteintech); TRX2 (Abnova); MitoTracker Red and Alexa Fluor 488 goat anti-rabbit IgG (MP); 4-hydroxy-2-nonenal (HNE) (ADI); 8-hydroxydeoxyguanosine (8-OHdG) (Millipore); nitrotyrosine (Sigma), and S-nitroso-cysteine (SNO-Cys) (Sigma-Aldrich).

### 2.2. Yeast two-hybrid screen

The human CERKL cDNA (1599 bp) was isolated from a HeLa cDNA library by nested PCR. An N-terminal truncated fragment of CERKL was cloned into the pGBKT7 vector and used as a bait. The plasmid was transformed into the yeast strain Y187. The pGADT7 fusion rabbit retina cDNA library was constructed and transformed into yeast strain AH109 following the user manual (Clontech). The library was screened by yeast mating; the cDNA insert from each positive clone was characterized by sequencing and Blast analysis.

### 2.3. Plasmid constructs and RNA interference

The full-length CERKL and TRX2 cDNAs were subcloned, respectively, into the pGEX-4T-1, pEGFP-C1 and p3xFLAG-CMV vectors. Truncations and missense mutants were constructed by PCR based on the wild-type gene and verified by sequencing.

Small interference RNAs (siRNAs) targeting different encoding regions of mouse CERKL were synthesized and purified by RiboBio (Guangzhou, China). CERKL siRNA #3 was used in the experiment. The targeting nucleotide sequences were as follows:

si-CERKL #1: 5'-GAGAAATACCGATGGATGT-3',  
si-CERKL #2: 5'-GGCAAACCATCCAGGGGCA-3',  
si-CERKL #3: 5'-GAGGAAGCCTGGAAGAAAT-3'.

The siRNA duplexes with non-specific sequences were used as siRNA negative control (si-NC).

### 2.4. Cell culture, co-immunoprecipitation and GST pull-down assays

NIH3T3 cells were grown in DMEM (Gibco) supplemented with 10% FBS. Cells were transfected with Lipofectamine 2000 (Invitrogen).

Cells were co-transfected with GFP-CERKL and Flag-TRX2 expression plasmids. After 48 h, cell lysates were harvested and immunoprecipitated with one antibody and protein G beads, and washed and analyzed by Western blotting with the antibody for the other proteins. The GST and GST fusion protein were expressed in *Escherichia coli* BL21 and purified using Glutathione-Sepharose beads (Thermo) following the manufacturer's protocol. After addition of cell extracts and incubation for 4 h at 4 °C, the bound beads were washed with PBS and analyzed by Western blot analysis.

### 2.5. Immunocytochemistry

Transfected cells were fixed in PBS/4% formaldehyde for 10 min at room temperature and permeabilized with PBS/0.5% Triton X-100 for 15 min. Cells were then incubated with the primary antibody (1:100–500) at 4 °C overnight and a fluorophore-labeled secondary antibody (1:2000) for 2 h. After staining with DAPI for 5 min, slides were mounted and viewed under a Leica TCS SP2 AOBS MP microscope system. The images were analyzed with Image J software.

### 2.6. Western blot and redox Western blot analysis

Cells were collected and lysed in RIPA buffer. Proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes (Millipore). The blots were incubated with primary antibodies (1:500–5000), followed by HRP-labeled secondary antibodies (1:20,000, Thermo). SuperSignal ECL substrate (Pierce) was used for the detection of signals.

For TRX2 redox analysis, cells were treated with CoCl<sub>2</sub> for the indicated time points. Cell lysates were incubated with 15 mM AMS (4-acetoamido-4'-maleimidylstilbene-2,2'-disulfonic acid, MP) at room temperature for 3 h and followed by Western blot analysis as described [24].

### 2.7. Detection of cell apoptosis

Cells were seeded in 6 well plates and transfected with indicated vectors or siRNAs. After 48 h post transfection, the cells were treated with 300 μM H<sub>2</sub>O<sub>2</sub> for 12 h. Following the user manual of Annexin V/PI apoptosis kit (MultiSciences), the cells were collected and resuspended in 0.5 ml Annexin V/PI binding buffer for 5 min in the dark and subjected to flow cytometric analysis for detection of apoptotic cells.

### 2.8. Animals and morpholino

All procedures were approved by the Institutional Animal Care and Use Committee at the Huazhong University of Science and Technology. Fertilized eggs of zebrafish were obtained and grown in incubators; embryos were analyzed at 3 dpf or 4 dpf, respectively. To knock-down zebrafish CERKL, a splice-blocking morpholino antisense oligonucleotide (5'-TCTGCTGTGATGTGAACGTACCGTT-3') target to exon2 was designed and synthesized by Gene-Tools. A standard mismatch morpholino was used as negative control. RT-PCR and Western blot analyses were performed to assay the CERKL transcription and expression when knocked down by splicing MO in zebrafish. The primer pair used for RT-PCR is F: 5'-GCCATCCATCTCAACAACCT-3', R: 5'-AAGAGC GGACCAACTTCATC-3'. Total RNA was prepared from 60 hpf fish treated with ZFCERKL-MO and controls; proteins isolated from 72 hpf fish were analyzed by Western blot analysis with an antibody for CERKL (Abcam).

### 2.9. Immunohistochemistry and histology

Frozen sections of 3 or 4 day zebrafish embryos were prepared as described [25]. The sections were incubated with PDT (PBS/1% DMSO/0.1% Triton) for 10 min and blocked with 10% normal goat serum in PBST (PBS/1% BSA/1% DMSO/0.1% Triton). The slides were incubated with a primary antibody at 4 °C overnight and a fluorophore-labeled secondary antibody (1:300 dilution) for 1 h at 37 °C. After staining with DAPI for 3 min, the slides were mounted in glycerol based anti-fade mounting medium for viewing. Apoptotic cells in the retina were detected using the Fluorometric TUNEL System (Promega). The sections were labeled with a TdT reaction mix for 1 h at 37 °C before viewing.

### 2.10. Statistical analyses

All experiments had been done at least three times. The statistical analyses were done with the Student's *t* test. Error bars in the graphs represent SD.

## 3. Results

### 3.1. CERKL interacts with mitochondrial TRX2

To understand the CERKL-mediated molecular mechanism in pathogenesis, we generated a cDNA library with mRNA isolated from rabbit retinas and constructed a two-hybrid library. We used the N-terminal of CERKL (1–165 aa) as the bait, and expression of the fusion protein was examined by Myc-antibody (Fig. 1A). After yeast mating screening, we obtained a positive clone that contained *rbTRX2*, which was the homologous gene of human *TRX2*. We identified *TRX2* protein as a potential CERKL interacting partner by yeast growth on the selective medium and X- $\alpha$ -gal assay in yeast (Fig. 1B). To confirm the interaction, we cloned the full-length human *TRX2* cDNA and constructed CERKL and *TRX2* eukaryotic expression vectors with different tags followed by reciprocal co-immunoprecipitation (IP) analysis. For NIH3T3 cells co-transfected with GFP-CERKL and Flag-*TRX2* vectors, the anti-GFP antibody but not IgG control could immunoprecipitate Flag-*TRX2* protein (Fig. 2A, left). The reverse IP with anti-Flag antibody likewise indicated that *TRX2* interacted with CERKL (Fig. 2A, right). To further confirm the interaction between CERKL and *TRX2*, similar results were obtained with reciprocal GST pull-down assays. We expressed GFP-CERKL or Flag-*TRX2* protein in NIH3T3 cells and mixed cell lysate with GST-*TRX2* or GST-CERKL fusion protein from *E. coli*, respectively. By using GST protein as negative control, GST-*TRX2* or GST-CERKL could pull down with GFP-CERKL or Flag-*TRX2*, respectively (Fig. 2B). These data suggested that CERKL directly interacted with *TRX2*.

While *TRX2* is known to locate in mitochondria specifically, a mitochondrial localization of CERKL has not been reported. To further validate the CERKL-*TRX2* interaction, we performed immunofluorescent studies in NIH3T3 cells. The endogenous CERKL co-localized with the

transiently expressed *TRX2*-RFP in a punctate, perinuclear fashion (Fig. 3A, upper panels). Moreover, co-immunostaining for CERKL and the mitochondrial marker Mito-Tracker, showed that the endogenous CERKL overlaps with mitochondria (Fig. 3A, lower panels). At higher magnifications, precise co-localization was observed (Fig. 3B and C). To further assess the association of CERKL with mitochondria, we isolated the mitochondrial and cytoplasmic fractions of NIH3T3 cells for immunoblotting, using tubulin and *TRX2* served as the cytosolic and mitochondrial marker, respectively. Results (Fig. 3D) confirmed the mitochondrial localization of CERKL. In total, results from our experiments showed that CERKL can localize to the mitochondria and interact with *TRX2*.

### 3.2. Pathogenic CERKL mutations disrupt the CERKL-*TRX2* interaction

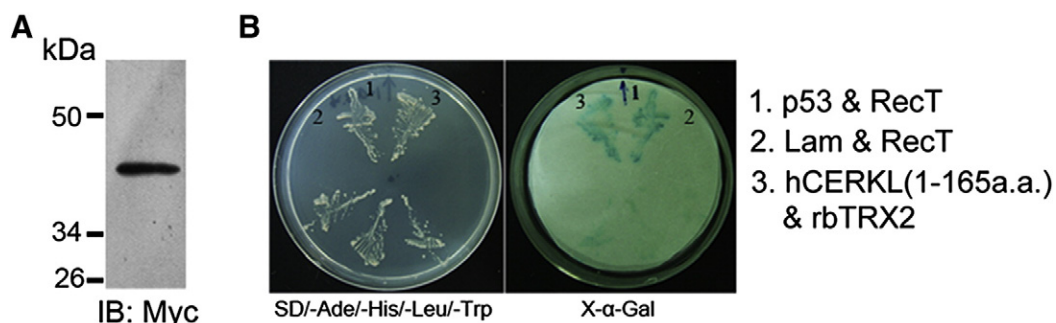
To fine-map the interaction domain of CERKL with *TRX2*, a series of GFP-tag N-terminal and C-terminal CERKL truncation vectors was constructed (Fig. 4A) in accordance with the deduced diacylglycerol kinase (DAGK) domain (165–368 aa) [15]. We expressed those GFP-CERKL proteins and mixed them with GST-*TRX2* protein. The GST pull-down results showed that: CERKL (1–165) and CERKL (40–165) clearly interacted with *TRX2*; CERKL (166–532) and CERKL (335–532) were unable to bind *TRX2* entirely; interaction of CERKL (100–165) and *TRX2* was reduced (Fig. 4B). These results indicated that the amino acids between 40 and 165 of CERKL are crucial for CERKL-*TRX2* interaction, and it is in accord with the bait used in our yeast two-hybrid screening.

Given that residues R106 and C125 were located in the *TRX2* interaction region of CERKL, we sought to determine if the point mutations R106S and C125W would affect the CERKL-*TRX2* interaction. The interaction between the mutants and wild type CERKL with *TRX2* were compared by GST pull-down assay, with GFP protein served as a negative control (Fig. 4C). For the same amount of GST-*TRX2* and GFP-fusion proteins, both the R106S and the C125W mutations significantly reduced the binding ability of CERKL and *TRX2* (Fig. 4D). These results suggested that the CERKL-*TRX2* interaction in the mitochondria has a critical physiologic function, which is disrupted by the pathogenic mutations R106S and C125W, respectively.

### 3.3. CERKL protects cells from oxidative stress-induced apoptosis

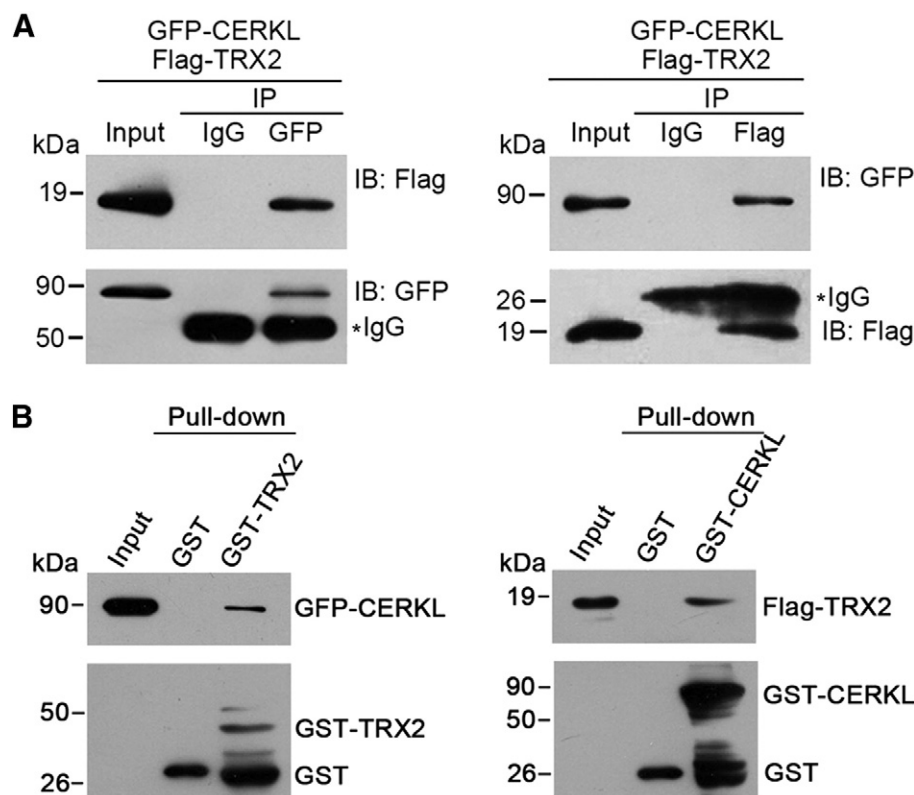
Apoptosis induced by oxidative-stress is a common cause of retina cell death [26]. Since *TRX2* plays a key role in the mitochondria-specific antioxidant system [21], our demonstration of a critical CERKL-*TRX2* interaction suggested that CERKL might protect cell from apoptosis induced by oxidative stress.

NIH3T3 cells were transfected with the indicated vectors, and cell death was detected by flow cytometric analysis after treatment with 300  $\mu$ M  $H_2O_2$ . Overexpression of CERKL caused a slight increase in vitality (from 72% to 84%) in basal condition (Fig. 5A). Under oxidative



**Fig. 1.** Identification of CERKL interaction with *TRX2* in yeast. (A) The bait fusion protein, BD-Myc-CERKL (1–165 aa), was expressed in yeast and analyzed by immunoblotting (IB) with anti-Myc (left). (B) Identification of *TRX2* as a potential CERKL interacting partner by yeast growth on the selective medium and X- $\alpha$ -gal assay. No. 1 is a colony of positive control, No. 2 is a colony of negative control which cannot grow on the medium, and No. 3 represents the *TRX2*-positive colony.





**Fig. 2.** Verification of the interaction between CERKL and TRX2 by co-IP and pull-down. (A) Reciprocal co-immunoprecipitation assays. NIH3T3 cell extracts transfected with GFP-CERKL and Flag-TRX2 were immunoprecipitated (IP) with the one indicated tag antibody and analyzed by immunoblotting analysis (IB) with the other antibody. Input, whole cell extracts. \*Indicates the nonspecific IgG band. (B) CERKL interacts with TRX2 in GST pull-down assays. Lysates from NIH3T3 cells expressing GFP-CERKL or Flag-TRX2 were incubated with GST-TRX2 or GST-CERKL solid beads, respectively. The proteins eluted by glutathione were subjected to SDS-PAGE and immunoblotting analyses with tag antibodies (top). The GST solid beads were used as a negative control. The GST-fusion proteins were analyzed by Western blot using anti-GST antibody (bottom).

stress, the live cells decreased from 72% to 21% in the empty-vector transfected cells, but cells overexpressing CERKL showed a higher survival rate, which is up to 48% (Fig. 5A).

Downregulation of CERKL in NIH3T3 cells was carried out using three siRNAs against different regions of CERKL. Suppression efficiency was checked by Western blot after 48 h post-transfection (Fig. 5B). The knockdown of CERKL greatly rendered the cells susceptible to H<sub>2</sub>O<sub>2</sub>-induced apoptosis; the percentage of live cells was reduced from 70% to 46%. However, without oxidative stress, suppressing CERKL had no obvious effects on cells (Fig. 5C).

These results collectively lend credence to the notion that CERKL protects cells from oxidative stress-induced apoptosis.

#### 3.4. CERKL maintains the reduced redox state of TRX2

The redox state of TRX2 reflects the regulation of TRX2 function [27]. To elucidate the biological implication of the interaction between CERKL and TRX2, we examined the redox state of TRX2 in the normal or CERKL-downregulated cells by redox Western blot methodologies, using 4-acetamido-4'-maleimidylstibene-2,2'-disulfonic acid (AMS). Separation of reduced and oxidized TRX2 bands on SDS-PAGE was achieved by a mass shift due to AMS alkylation of thiols and the bands were identified by immunoblotting with anti-TRX2 antibody. As shown in Fig. 6, suppressing CERKL had no significant effect on the reduced form of TRX2 under normal conditions. Cobalt chloride (CoCl<sub>2</sub>), induces reactive oxygen species (ROS) generation, leading to cell death [28,29]. When CERKL was silenced and the cells challenged with 0.5 mM CoCl<sub>2</sub> for 12 h, almost all the TRX2 protein changed to the oxidized form. Quantifying the band intensities showed that the Re/Ox TRX2 level was dramatically decreased in contrast to the control cells (Fig. 6).

This result therefore indicated that CERKL plays an important role in modulating the TRX2 redox state and the protective effects of CERKL are likely mediated by maintaining TRX2 in a reduced form.

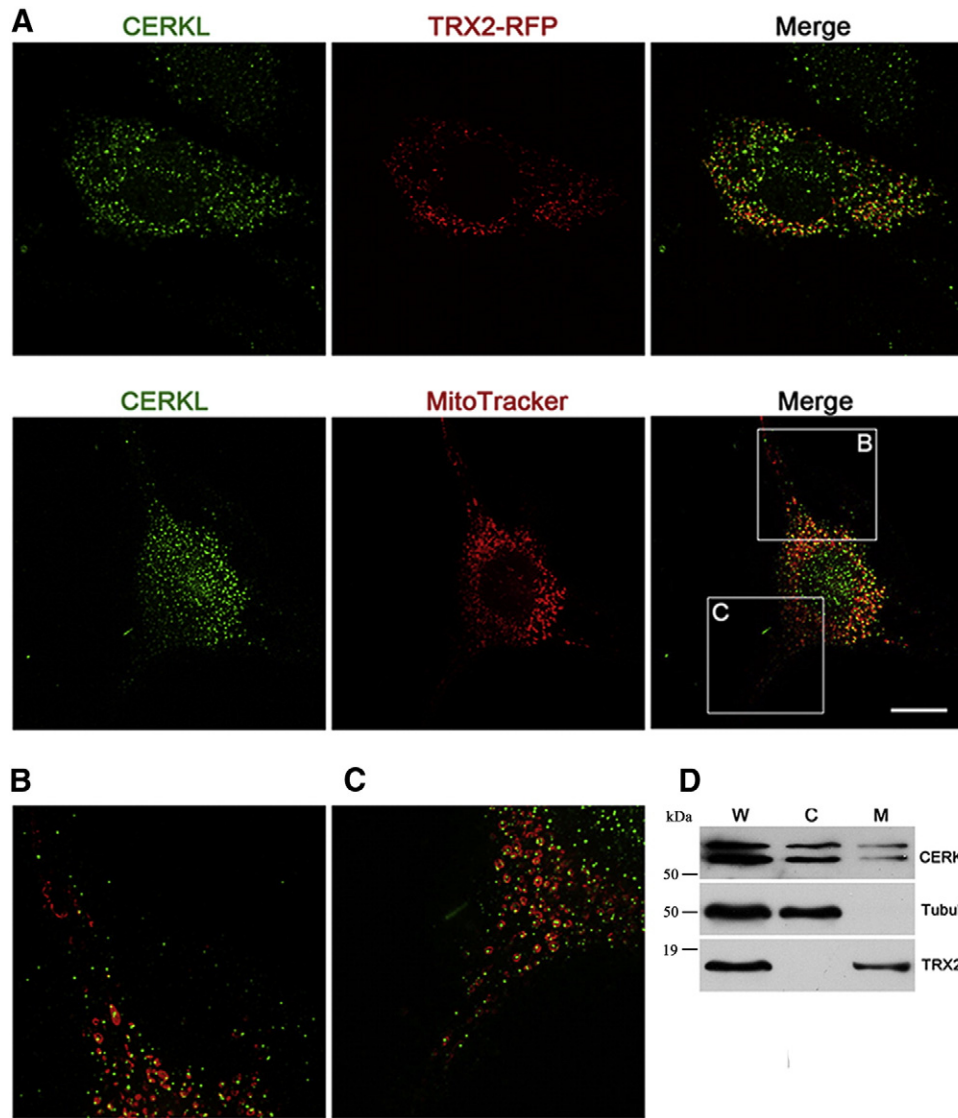
#### 3.5. ZFCERKL deficiency causes zebrafish retinal degeneration and photoreceptor apoptosis

To analyze the critical role of CERKL in the pathogenesis of retinal degeneration *in vivo*, we studied CERKL in a zebrafish model [30]. We identified an orthologous gene of the human CERKL in zebrafish (Genbank accession no. BC139548). Immunostaining was performed on frozen sections of a 4 dpf wild type zebrafish retina. A strong signal was seen in the outer segment (OS) and inner segment (IS, arrow) of the photoreceptors; fainter signals were observed in the outer plexiform layer (OPL), inner nuclear layer (INL) and ganglion cell layer (GCL) (Fig. 7A). The distribution of ZFCERKL in zebrafish retina was in agreement with previously published data in mice [13,14,31].

We suppressed the expression of ZFCERKL by injecting a splice-blocking morpholino in zebrafish embryos. Both mRNA and protein expression of ZFCERKL were reduced in MO-zebrafish compared to the injection of control morpholino (CMO) (Fig. 7B). To test if ZFCERKL deficiency may cause photoreceptor apoptosis, TUNEL staining was performed on frozen sections of control and defective retinas from 4 dpf zebrafish. Similar to recently reported data by Riera et al. [32], our results clearly showed TUNEL positive cells in the photoreceptor layer and inner nuclear layer of ZFCERKL-MO zebrafish (Fig. 7C).

#### 3.6. Deficient ZFCERKL caused oxidative damage in zebrafish retina

To further investigate if a deficiency of ZFCERKL would lead to peroxidation in the zebrafish retina, a series of oxidative damage biomarkers



**Fig. 3.** CERKL is localized in mitochondria where it associates with TRX2. (A) Endogenous CERKL co-localized with TRX2 in mitochondria shown by confocal microscopy. NIH3T3 cells transfected with TRX2-RFP (red) were immunostained with CERKL (green) antibody (upper panel) and cells co-immunostained with CERKL (green) antibody and mitochondrial indicator MitoTracker (red) (lower panel). Scale bar = 10  $\mu$ m. (B and C) Viewed with higher magnification (marked with white squares in A). (D) Immunoblotting determined that the endogenous CERKL does localize in mitochondria in addition to the other subcellular organelles. Cytoplasmic and mitochondrial fractions of NIH3T3 cells were isolated and determined by indicated antibodies. W, whole cell extracts; C, cytosol; M, mitochondria.

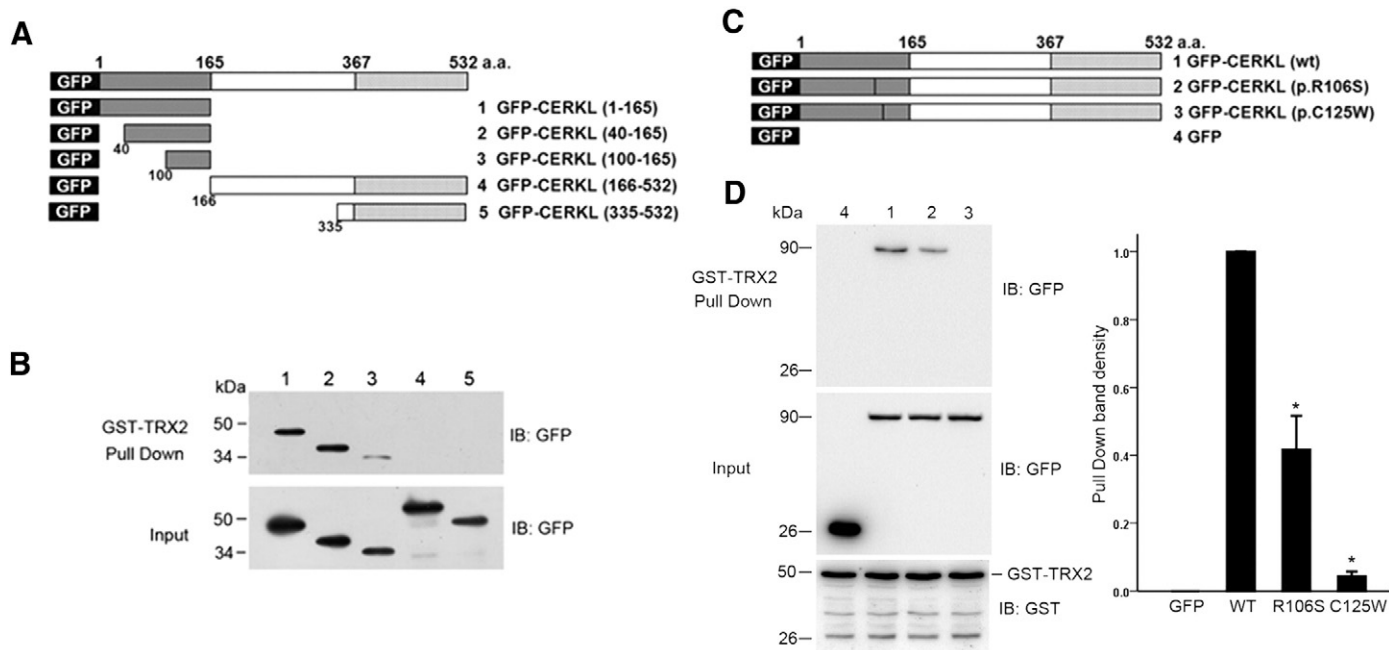
were used in immunohistochemical staining. The 4-hydroxy-2-nonenal (HNE) is a major product of endogenous lipid peroxidation and a useful biomarker of lipid peroxidation in retinal diseases [33,34]. Immunostaining for HNE resulted in faint fluorescence throughout the retina in control 4 dpf zebrafish (Fig. 8A upper panel). The *ZFCERKL*-MO zebrafish showed strong hyperfluorescence only in the photoreceptor OS/IS area of the retina (Fig. 8A lower panel). Similarly, 8-hydroxydeoxyguanosine (8-OHdG) has been used as an indicator of oxidative DNA damage [35]. Staining of control-MO zebrafish retina with the anti-8-OHdG antibody showed mild background staining, particularly along the inner plexiform layer (Fig. 8B upper panel). In contrast, a section from *ZFCERKL*-MO showed prominent staining in all the retinal layers, especially in the photoreceptor OS/IS layer and axons (outer plexiform layer) (Fig. 8B lower panel). In addition, S-nitroso-cysteine (SNO-Cys) and nitrotyrosine have been served as the indicators for protein oxidative damage by nitric oxide (NO) in cells. S-nitrosylation of cysteine thiols and peroxynitrite of tyrosine residues in proteins are the important effectors in NO-derived signal transduction pathways [36,37]. Confocal microscopy

of an SNO-Cys-stained section from 4 dpf *ZFCERKL*-MO zebrafish showed prominent staining of the photoreceptor OS/IS and axon layers compared to the mild staining seen in the OS/IS region of the control retina (Fig. 8C). The pattern of staining for nitrotyrosine was similar to that seen for SNO-Cys. After silencing *ZFCERKL*, the retina showed much more punctate staining in photoreceptor OS/IS and INL in contrast to the control retina (Fig. 8D). The above results clearly indicated that *ZFCERKL* was essential for protecting the retina from oxidative stress.

In sum, the *in vivo* results from our studies in zebrafish support the conclusion that a normal physiologic function of CERKL is to protect retinal cells from oxidative stress-induced apoptosis, especially of cone and rod photoreceptors.

#### 4. Discussion

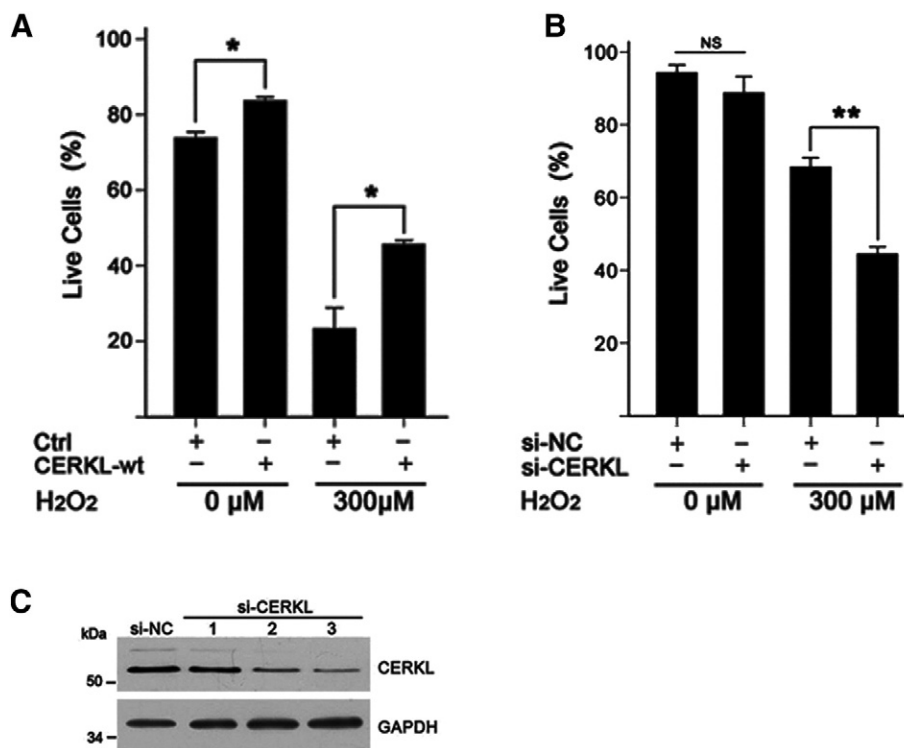
Our *in vitro* and *in vivo* studies have revealed that CERKL protects cells from oxidative stress by modulating the redox state of TRX2 via protein–protein interaction. These results provide a mechanistic explanation of how mutations in *CERKL* cause the death of photoreceptors. To



**Fig. 4.** Mapping the key domains of CERKL that interact with TRX2 and the effects of CERKL pathogenic mutations on this interaction. (A) Schematic model of truncated CERKL. (B) Mapping the interaction domains of CERKL with TRX2 by GST pull-down assays. NIH3T3 lysates expressing the indicated GFP-CERKL were applied to GST-TRX2 protein beads respectively. The 40–165 residues of CERKL are required for interaction with TRX2. (C) Schematic representations of wild type and two missense mutations of CERKL. (D) GST pull-down assays determined that CERKL pathogenic mutations can disrupt the interaction. Equal protein loading of wt (wild-type), R106S, C125W CERKL and GST-TRX2 was determined by immunoblotting with anti-GFP or anti-GST, respectively. \*p < 0.05.

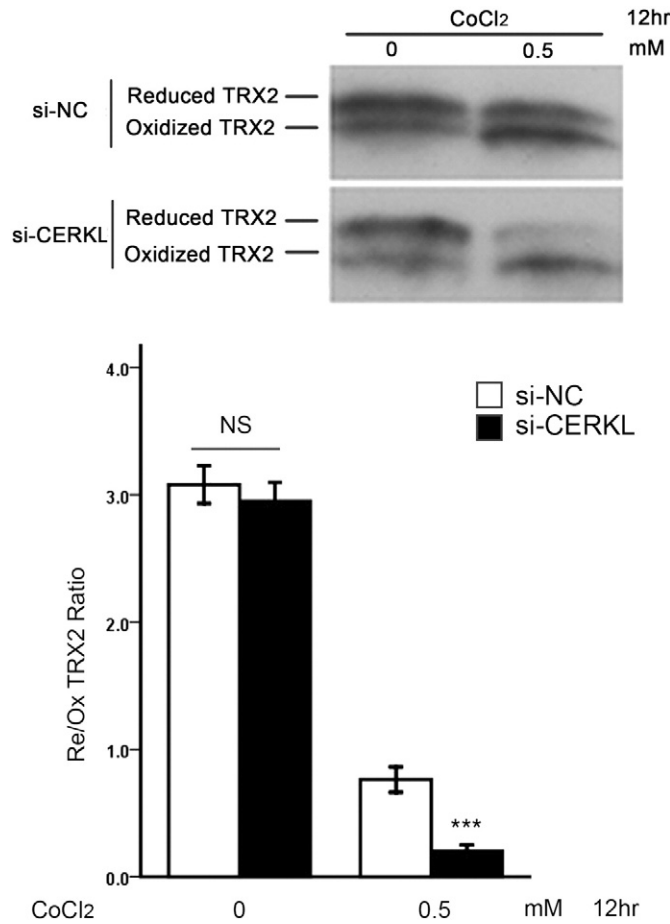
our knowledge, this is the first demonstration that CERKL acts as a key player in the mitochondrial thioredoxin-mediated antioxidant pathway in the retina.

Tuson et al. reported that overexpression of CERKL protects cells from apoptosis when challenged by hydrogen peroxide [38]; here we have provided the evidence that CERKL displays an important



**Fig. 5.** CERKL protects NIH3T3 cells from oxidative stress induced apoptosis. (A) Overexpression of CERKL protects from oxidative stress-induced apoptosis. Cells were transfected with the indicated vectors for 48 h and treated with or without H<sub>2</sub>O<sub>2</sub> for 12 h, and analyzed by flow cytometry. Percentages of live cells were shown in the histogram. (B) SiRNAs suppressed CERKL expression. Cells were transfected with three different CERKL-specific siRNAs or scrambled siRNA (NC) for 48 h. Endogenous expression of CERKL was examined by Western blot. #3-siRNA-CERKL exerted the highest efficiency. (C) Knockdown of CERKL render cells more sensitive to oxidative stress induced apoptosis. Cells were transfected with the indicated siRNAs for 48 h and treated with or without H<sub>2</sub>O<sub>2</sub> for 12 h, and analyzed by flow cytometry. Percentages of live cells were shown in the histogram. Experiments were done in triplicates. \*p < 0.05; \*\*p < 0.01; NS, not significant.



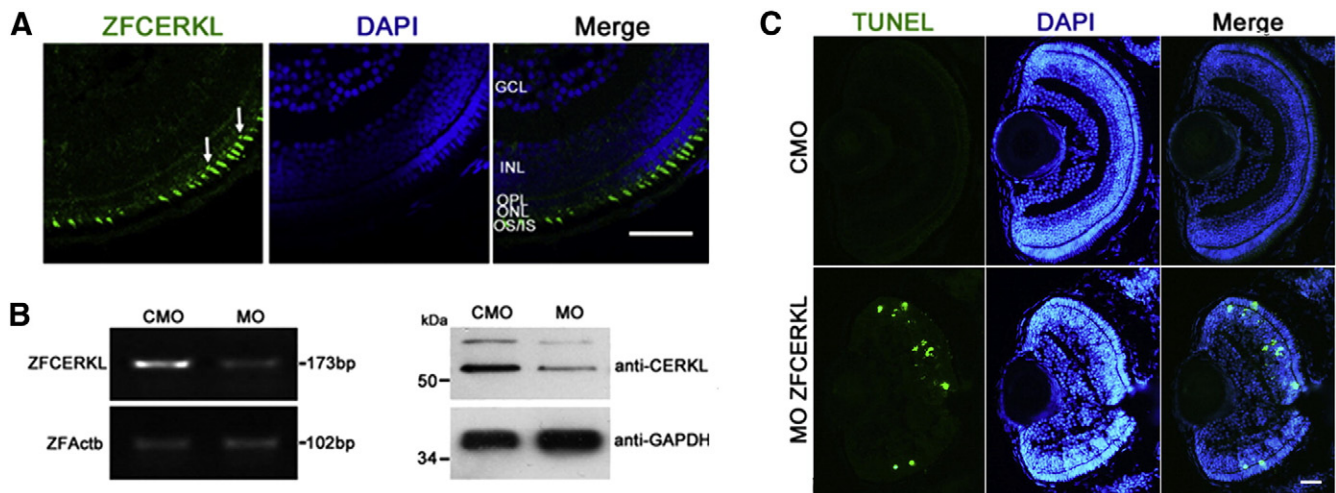


**Fig. 6.** CERKL modulates TRX2 oxidation. CERKL is essential for maintaining the reduced state of TRX2. NIH3T3 cells transfected with the indicated siRNA were exposed to CoCl<sub>2</sub> for the indicated time and concentration. Separation of reduced and oxidized TRX2 bands is performed by AMS-redox immunoblotting analysis. Quantified band intensities of reduced versus oxidized TRX2 from three independent experiments are shown below. \*\*\**p* < 0.001.

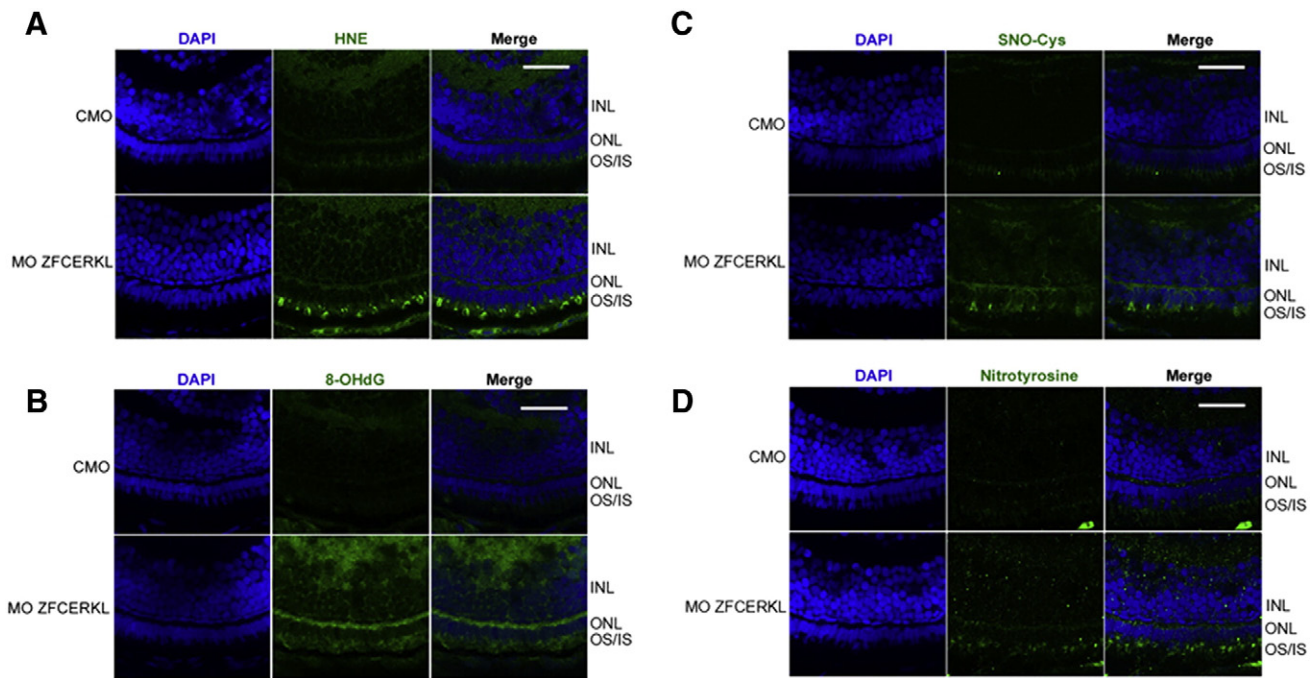
anti-apoptosis function by modulating the mitochondrial peroxiredoxin-mediated antioxidant pathway. Mitochondria are the main source of cellular ROS and play an important role in the signal transduction of

apoptosis [39–41]. The cell antioxidant systems, including glutathione peroxidases and peroxiredoxins, maintain the generally low levels of cellular ROS; the former depend on glutathione for their reduction whereas the latter on TRX2 [41]. Many reports have illustrated the role of TRX2 on mitochondria-mediated apoptosis [21,42,43]. We have demonstrated that CERKL defect and/or deficiency lead to a shift in redox equilibrium of TRX2, which in turn causes oxidative damage and apoptosis of retinal cells. TRX2, together with thioredoxin reductase-2 (TrxR2) and NADPH, forms the mitochondrial specific thioredoxin system. TrxR2 utilizes NADPH to catalyze the conversion of oxidized TRX2 into a reduced form. By direct protein–protein interaction, CERKL maintains the reduced redox state of mitochondrial TRX2. Pathogenic consequences of CERKL deficiency may result from an increase in the oxidized form of TRX2. While our data do not provide detailed information on how CERKL might facilitate the redox reaction and its role in TrxR2 activities, our results do suggest that CERKL modulates TRX2-mediated downstream pro-apoptotic pathways using the redox state of TRX2 as a switch. This speculative proposal of the detailed mechanisms connecting the reduced form of TRX2 to ROS scavenging and prevention of oxidative stress-induced retinal cell death can be tested in future experiments.

The highly complex expression and distribution patterns of CERKL in mouse and human have been reported. CERKL proteins were reported to be mainly localized in the endoplasmic reticulum and Golgi compartments, and may shift localization to nuclei and nucleoli [38]. Here, we demonstrate that some endogenous CERKL may distribute in mitochondria. Our result may be consistent with Vekslin et al. Their finding revealed that CERKL is highly concentrated in the perinuclear region in retina-derived cell lines [14]. The complex subcellular location of CERKL suggests that interaction with TRX2 in mitochondria to prevent oxidative damage may only be one of several functions of CERKL [14, 38]. For example, CERKL is also localized to the ER. It was discovered recently that the thioredoxin-interacting protein (TXNIP) is a critical signaling node that links ER stress to the death of beta cells during the progression of diabetes [44,45]. In the ER, CERKL might have roles in antioxidant function, in a manner analogous to TXNIP. Thus, our results also perhaps extrapolated to support a model that in the different subcellular locations, the function of CERKL is in the prevention of oxidative stress-induced retinal cell death; the pathways involved may be different, but the outcome of disrupting CERKL function—oxidative stress—is the same. This notion is consistent with the common clinical phenotype associated with the different pathogenic mutations



**Fig. 7.** Expression pattern of ZFCERKL in the zebrafish retina and silencing ZFCERKL resulted in retina degeneration. (A) Frozen section of zebrafish retina was immunostained with CERKL polyclonal antibody. ZFCERKL showed a strong signal in the outer segments (OS) and inner segments (IS, as indicated by white arrows) of the photoreceptor cell layer and a weaker signal in the outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL) and ganglion cell layer (GCL). (B) Transcription and expression of ZFCERKL were knocked down by splicing MO in zebrafish. The fish were subjected to RT-PCR (left) and Western blot (right) analysis respectively. (C) TUNEL staining showed that knocking down ZFCERKL expression resulted in the apoptosis of retinal cells (green).



**Fig. 8.** ZFCERKL is essential for protecting zebrafish retina from oxidative damage and modulating ZFTRX2 redox states. Silencing ZFCERKL resulted in oxidative damage in zebrafish retina. Peroxidation of lipids (A), DNA (B) and proteins (C and D) were indicated by immunofluorescent staining with HNE, 8-OHdG, SNO-Cys and nitrotyrosine antibodies respectively. In addition to the irregular arrangement of photoreceptor nuclei, all of them had strong immunostaining in the retina of 4 dpf ZFCERKL-MO zebrafish, compared to faint staining in 4 dpf control zebrafish retina. INL, inner nuclear layer; ONL, outer nuclear layer; OS/IS: outer and inner segments of photoreceptors. Nuclei were stained with DAPI. Scale bar = 25  $\mu$ m.

of *CERKL*. Whether the clinical label is RP or CRD, the presentation includes severe early macular involvement and clumped pigmentation. Both rod and cone functions are diminished early. These specific common phenotypic features suggest an underlying common disease mechanism. Therefore, the *CERKL*–TRX2 antioxidant pathway provides a potential explanation for the mechanism by which different pathogenic mutations in *CERKL* might lead to the common disease phenotype in human. In other words, our results suggest the hypothesis that the biological functions of *CERKL*, regardless of its subcellular location, maybe be related to antioxidant activities.

In *CERKL* knockout and knockdown mice, unlike in zebrafish, only mild phenotypes were observed in the retina. The species-specific transcriptional start sites and products in the retina might explain the lack of the anticipated phenotype in mouse [16,17]. Alternatively, *CERKL* is highly expressed in cone photoreceptors, with a very low expression level in rods [14]; the full manifestation of photoreceptor degeneration in defective or deficient *CERKL* may require a high density of cones, such as in the human macula and the zebrafish retina. In comparison, the density of cones is low in the mouse retina, where only 3% of photoreceptors are cones [46]. Independent of the basis for the species difference, positive results in zebrafish give an impetus to further development of this animal model for analyzing the function of *CERKL*.

The single function of photoreceptors is vision, which is achieved by elegantly orchestrated complex biochemical cascades of phototransduction, visual cycle and synaptic transmission. Accordingly, photoreceptors are the highest energy consuming cells in the human body [1,3]. Near constant exposure to radiation from sunlight promotes generation of ROS in rods and cones. These environmental and intrinsic challenges make them highly susceptible to oxidative damage. ROS from mitochondria are toxic by-products of metabolism with the potential to cause damage to lipids, proteins and DNA. Our data suggests that macromolecules in the retina show evidence of oxidative damage in *CERKL* knockdown zebrafish. Since ROS damage is involved in a broad category of retinal degenerations, our linkage of the underlying disease mechanism of a type of cone–rod photoreceptor degeneration to a

defect in the TRX2-dependent and peroxiredoxin-mediated antioxidant pathway may provide rational targets for new therapeutics of retinal degenerations [6,47].

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